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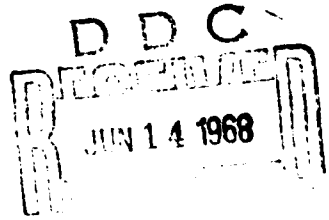

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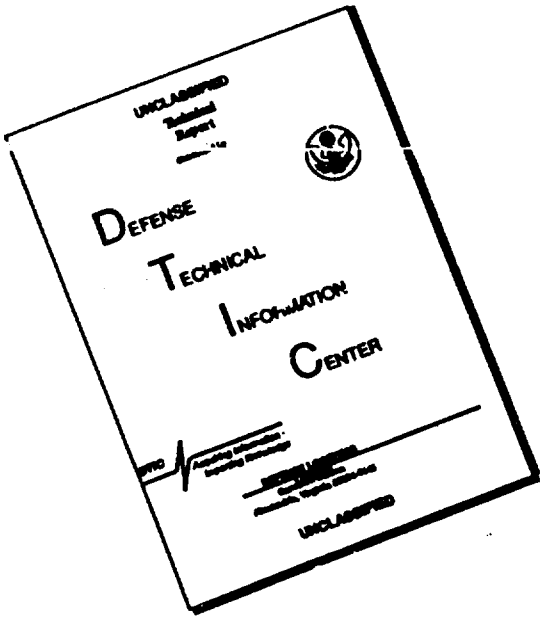

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THE VIRULENCE OF PURINE-AUXOTROPHS OF BACILLUS ANTHRACIS

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U. S. ARMY

FORT DETRICK, FREDERICK, MARYLAND

THE VIRULENCE OF PURINE-AUXOTROPHS OF BACILLUS ANTHRACIS

(Following is a translation of an article by G. Ivanovics and Elizabeth Marjai, University of Szeged, Hungary, published in the German language periodical, Zent. Bakt., 193, 1964, pages 363-75. Translation performed by Constance L. Lust.)

The minimal nutritional requirements of Bacillus anthracis can be met with an amino acid mixture, salts and glucose (Gladstone 1939). This pathogen requires no exogenous vitamins and nucleic acid-bases, even though they do enhance the development of its cultures (Brewer and Mitarb 1946). Previously we reported that under mutagenic influences mutants can be isolated of cultures, which require additional bases or certain vitamins (Ivanovics, Varga and Marjai 1963). In the course of our investigations we have isolated and studied 186 such auxotrophic mutants with special requirements (Ivanovics, Varge and Marjai 1964). More than half of the auxotrophs required some purine base. These purine-auxotrophs could be divided into six groups according to whether they required single bases, as adenine, guanine, hypoxanthine or xanthine, or combinations of bases. In four of the groups, Pu 1, Pu 2, Pu 3, Pu 4 do novo purine synthesis appeared to be identical to that of Enterobacteriales (Mugasanik 1962).

The place of the presumed block in the four groups of our auxotrophs is illustrated in Figure 1. It should be noted that with the components of group Pu 1, where base synthesis proceeds from ribose-5-phosphate in 11 steps to inosinic acid, the block could occur in several places. The genotype of the mutants which can be satisfied with hypoxanthine could be different. A place for the block will be considered which applies for the mutants only.

The auxotrophs were isolated from an acapsular mutant of one of our laboratory strains (Vollum-strain). Since the original work of Preisz (1904) it is known that the capsul has a basic role in the virulence of B. anthracis. While virulent strains exist as encapulated bacteria in 5-25% CO_2 and form mucoid colonies (Nungester, 1929, Sterne 1937, Ivanovics, 1937), the acapsular strains do not produce no capsuls under these conditions. Newer investigations (Thorne, 1960, Meynell 1963) have shown in the populations of acapsular strains (C-) small numbers of encapsulated C^+ mutations (reverted) can occur. The auxotrophs we used were isolated from acapsular (VC^-) mutants of the Vollum strains. The goal of this study was to produce encapsulated mutants of purine-auxotrophs and to prove their virulence. During this study we found that the members of group Pu 4-auxotrophs, whose purine requirement was met by adenine, were completely non-pathogenic. With these studies we hoped to be able to comprehend the biochemical basis of one, or more of the factors which play a role in the pathogenicity of B. anthracis.

Methods

Medium was prepared essentially as described earlier (Ivanovics and coworkers 1964; Ivanovics 1962). YP= yeast extract peptone medium; BCM = vitamin free casein-hydrolysate basal medium plus thiamin. This is identical to MCM (Ivanovics et al 1963); YC = yeast extract-casein hydrolysate medium.

Determination of the requirements of the auxotrophs

The auxotrophs were maintained in the form of spores and were used as inoculum. Their base requirement was determined by using all four bases (10 ug/ml) in BCM-agar and inoculating this. Quantitative determinations of nutritional requirements were performed using BCM solutions and growth was expressed as optical density. (Growth as it was dependent on the concentration of bases).

Investigation of capsul formation

YC-agar plates containing $NaHCO_3$ were inoculated and incubated in 25% CO_2-air (Invanovics 1962). Isolation of encapsulated (C^+) mutants from acapsular (C^-) strains in vitro was done similar to the method of Meynell (1963): (10^{10} ml. of the YC-agar (50°C) containing HCO_3- was mixed with 2 ml WaC-phage-lysate (Ivanovics 1963) (10^{10} per ml of plaque formers) and poured into plates. The plates were inoculated with a heavy suspension of spores of the acapsular strains(10^7-10^8 spores per plate) and the cultures were put CO_2-air. The mucoid colonies were suspended in anti-Wa phage solution and subcultured similarly. The strains obtained were not lysogenic.

Virulence was determined by inoculating mice subcutaneously with the spore-material. The bacteria were isolated from heart blood of dead animals, and their nutritional requirements checked with BCM-agar.

Results

The purine-auxotrophs investigated and their requirements.

For these trials the strains described were chosen (Ivanovics and coworkers 1964). The strains were in three groups (Pu 1, Pu 3, Pu 4); no "leaky" strains were included. They were marked (designated) by their isolation number, behavior of capsul formation and nutritional requirements. Based on their food requirements the following points arose.

hy-; The bloc is found befor inosinic acid synthesis and its requirements are satisfied with all four bases (adenine, guanine, hypoxanthine, xanthine).

ad-; requires specifically adenine

gu-; utilizes only guanine

Further, the strains can be characterized as follows: The hy⁻ auxotrophs can utilize inosine, adenosine and deoxyadenosine as well as the four bases. Adenylic acid or inosinic acid were not utilized (or only very minimally). This may indicate a connection with the minimal decomposition of nucleotides.

The requirements of the ad-strains could be met with nucleosides of the bases as well as with adenine, but did not utilize nucleotides. A similar phenomenon was exhibited by the gu-auxotrophs.

The hy-strains behaved somewhat more variably in quantitatively utilizing the individual bases, especially in reference to the speed of utilization (table 1). The conditions varied from one case to another, as may be seen in the table using strains 61 C⁻hy⁻ as an example.

The ad⁻ auxotrophs required somewhat greater quantities of the base than the other purine-auxotrophs. An insight of the growth-reactions of the one strain is presented in figure 2.

Virulence of acapsular strains. The single factor of the virulence of B. anthracis is capsule formation. Acapsular mutants kill animals only after very high numbers of organisms are present. At the sight of injection in the animal rather large oedemas form, from which the infection spreads and eventually kills the animal. Bacteriological investigation of blood gives evidence of non-encapsulated bacteria (Sterne 1937; Stamatin 1937; Ivanovics 1938). More recent reports found that in populations of acapsular strains encapsulated mutants may also be present (Thorne 1960; Meynell 1963).

The capsular mutants may already be present in the inoculum, or they could arise during the course of the limited multiplication of the acapsular bacteria within the animal. Through selection the typical picture of anthrax results with its usual bacteriological observation.

When mice were inoculated with 10^6 spores of the parent acapsular Vollum strains (VC^-) they lived. When quantities of 10^7-10^8 were used a part of them died. As a rule the encapsulated reverted mutants (VC^+) were isolated from their heart blood. Sometimes VC^- and VC^+ bacteria were observed next to each other in the blood of the animals; othertimes only VC^- were found during bacteriological studies. According to Thorne (1960) the death of the animals may be due to a toxin effect in the case of the acapsular mutants.

In order to ascertain whether the acapsular mutants exhibit some pathogenic properties we have injected large numbers of mice. We did not inject spores, but rather injected the vegetative forms intraperitoneally. 24 hours cultures prepared in YP-agar were used as thick suspensions in saline and employed for injection; 0.5×10^7 to 2×10^8 colony forming units were inoculated. The suspensions of the strains contained bacteria in chains of varying length. The results of the trials performed with various purine-auxotrophs are summarized in table 2.

It can be seen that none of the 56 animals inoculated with the ad-strains died, whereas many of those injected with the other auxotrophic groups died. The strains isolated from heart blood of those animals all retained their original auxotrophic properties. The isolated bacteria (from heart blood) could be different however, corresponding to the individual animals. In a considerable number of them mucoid (encapsulated) bacteria were found; in other cultures only acapsular bacteria were observed, and in still other cases both encapsulated and acapsular bacteria were present simultaneously.

In the case of strain $30C^{-}gu^{-}$ no encapsulated bacteria could be isolated from any of the animals. It could have been that this strain did not revert (Meynell 1963) but it was shown that an encapsulated variety could be formed in vitro.

Isolation of encapsulated auxotrophs in vitro. After all mice infected with mutant ad- remained alive. We hoped to obtain encapsulated variants from them via phage-plates. In this way encapulated strains could be obtained from all the strains we studied. When the phage-plates were inoculated with spores, we obtained a proportion of $1/10^{6}$ mucoid colony-forming-units. These encapsulated strains 6 C^{+} ad-, 20 C^{+} ad-, 23 C^{+} ad- and 83 $C^{+}ad^{-}$, retained their specific requirements for adenine.

In this way encapsulated mutants could be isolated from other auxotrophs, namely strains 5 $C^{+}hy^{-}$, 58 $C^{+}hy^{-}$, 61 $C^{+}hy^{-}$, as well as 30 $C^{+}gu^{-}$. In further experiments we used as encapsulated auxotrophs the strains obtained in vitro, because in the isolation of this clone the effect of selection on virulence was not involved.

From strain 23 $C^{+}ad^{-}$ a protrophic reverted mutant could be made. DCM-agar plates were inoculated with large numbers of spores of the encapsulated auxotrophs. Prototrophs isolation was attempted in this way. Prototrophic reverted mutants could be obtained in a proportion of 10^{-7} from the strain. This(prototroph) appeared to be identical in phenotype to the wild strain VC^{+} in capsual formation as well as in nutritional requirements.

It appears that capsule formation is completely independent of the phenotype of the individual auxotrophs. This may mean that in the mutants we studied, capsule formation is not related to the genetic defect in purine synthesis.

Virulence of encapsulated purine-auxotrophs

Virulence was determined via subcutaneous inoculation of mice with spore suspensions. The control material was the wild strain VC and the reverted mutant $23C^{+}ad^{+}$.

With very few exceptions, mice infected with 10 spores of strain VC^{+} died. In order to illiminate sources of experimental error 20 spores were considered as the smallest lethal dose (DLM).

Groups of 6 mice weighing 24-28 g were infected with 2 x 10, 2 x 10^2, 2 x 10^3 spores respectively. In certain cases 10^4, 10^5, 10^6 were used. The infected animals were placed into two groups. One-half received 1 mg of the corresponding bases and later 0.5 mg intraperitoneally at 12 hour intervals. The observations were carried out over three weeks. All dead animals were "worked up" bacteriologically; their heart blood was inoculated on YP-agar, and the nutritional requirements of each strain were checked. The dose that killed 2/3 of the animals was considered to be the DLM (minimum lethal dose). Only dead animals with positive bacteriologicak findings were then considered for anthrax. Sometimes the isolated strains were not auxotrophs, but rather prototrophs. This was evaluated correspondingly. The experiments were done at least twice and the results combined. The data represent average results. In cases of completely identical results the range is listed.

One difficulty encountered with the bases was that adenine became toxic after several injections. Therefore, a maximum of 5 injections (totally 2.5 mg in 3 days) was used. The effect was considered toxic when the dead animals showed no pathological changes (oedems) and the bacteriological picture was negative.

The difficulty with guanine was that it was somewhat insoluble in water; it was injected as a water-suspension.

Only two animals died of 75 which had been infected, with doses of 2 x 10^3-2 x 10^6 of the four encapsulated strains but not treated with adenine.

The bacteriological report was negative for both. That meant that death was not attributable to the infection, eight animals died of 58 which had been infected with 2 x 10^3-2 x 10^6 spores. This had been repeated with adenine treated mice. Sections and bacteriological reports were negative in 7 cases; one case had both reports positive. The strain cultured out was not ad^-, but a prototrophic reverted strain. In one experiment the animals received besides adenine simultaneously an equal amount of hypoxanthine. Thirty six mice had received spores of the four ad-strains (10^3-10^5 colony formint units). Four animals died one with negative bacteriology. The prototrophic variants could be isolated from the blood of the other three. The ad^- strains were non-pathogenic when 5 mg histidine was given along with adenine.

In contrast all of 10 animals died when inoculated with 2 x 10 spores of 23 C^+ ad^+. This was similar to the wild strain.

The results of the encapsulated, adenine requiring strains showed the following. Of 185 animals inoculated with 200-2 x 10^6 spores (not treated with adenine) none died due to the pathogenicity of the ad-auxotrophs.

On the other hand the strains of phenotype hy^-, and gu^-, after enrichment with the corresponding base-or even without base-proved to be pathogenic in mice. Seventy two mice were infected with the three

hy⁻ strains (2^{0}-10^{6}) and half were treated with hypoxanthine. Of the treated mice 27 died; of the untreated 25 died. All dead mice had a positive bacteriological report. Seventy eight animals were similarly treated with gu⁻ strains. Of 78 animals infected with 200-2 x 10^{5} spores 57 died.

Table 3 presents a general picture of the experiments which were performed using 8 encapsulated auxotrophs.

The original (VC^{+}) strain and that isolated from a revertant 23 $C^{+}ad^{+}$ adenine-auxotroph $23C^{+}ad^{+}$ proved to be equally virulent. The ad^{-} mutants even at levels of 10^{6} were not lethal for mice. The adenine treatment did not influence the infection in this case. The hy⁻ auxotrophs were very virulent even when not treated with bases. One strain did exhibit increased virulence after repeated addition of the bases. Auxotrophs of gu⁻ showed similar behavior, but these caused lethal infections even without this.

Discussion of Results

It is interesting that one group of purine-auxotrophs of B anthracis, namely one which is dependent on adenine, is completely non-pathogenic for mice. The results seem to indicate that the apathogenesis of these mutants cannot be explained with the adenine requirement of the organism. These bacteria do not multiply in vivo, even if the 25 gram mice are given 1 mg adenine intraperitoneally for two or three days after the infection. The adenine requirement of these strains can be met in vitro with 10-15 ug/ml of base. Greater quantities of adenine could not be used because of its toxicity.

There are other examples, that adenine-auxotrophs of certain pathogens, like Salmonella typhi and Pasteurella pestis, can kill the animals they infect, especially when the infection is combined with adenine supplementation. (Burrows 1955). These examples support that the non-pathogenicity of the adenine-auxotrophs of B. anthracis cannot be attributed to lack of adenine, but must be related to some other basic factor. Our results with the purine auxotrophs support this concept. The mutants multiply in vivo and their virulence is fully manifested if corresponding purine derivatives are administered after the infection.

The "non-pathogenic" property appears to be typical for the adenine auxotroph of B. anthracis. Besides these purine-auxotrophs the thermosensitive guanine-auxotrophs (Ivanovics in press), as well as pyrimidine and vitamin auxotrophs (unpublished results) are also pathogenic.

In our adenine auxotrophs the bloc in purine synthesis seems to be related to stopping pathogenesis. The properties of the mutants we studied and their interrelationship is presented schematically in figure 3.

As can be seen, virulence is also determined by the adenine defect, independently of the capsule. Reversion of the capsule-lacking mutation

restores full virulence. Since capsule formation (synthesis) and adenine synthesis are both required equally for virulence, it is understood that the acapsular adenine-auxotrophs cannot kill mice. It is unlikely that a simultaneous reversion of both interdependent mutations could occur in our case.

In purine biosynthesis inosnic acid is changed (hypoxanthin-nucleotide) in two steps (Abrams and Bentley 1955) (see fig. 1). Inosinic acid is condensed with asparigine to adenylosuccinate in the presence of adenylosoccinate synthetase (Lieberman 1956) and this product is transformed into adenylic acid and fumaric by adenylsuccinase. This enzyme interestingly plays a role earlier in purine synthesis; in the formatiom of the pyrimidine ring be breaking 5-amino-4-imidazol-N-succinocarboxyamide-nucleotide into 5-NH_2-4-imidazol-carboxyamide-nucleotide and fumaric acid (Miller, Lukes and Buchanan 1957). This is a special phenomenon in biosynthetic processes since the same enzyme acts on different substrates. The same segment of the chromosome controls two widely seperated biochemical reaction sequences.

Thus far we have not been able to explain which of the two enzymes involved in forming adenylic acid from inosinic acid is impaired in our adenine auxotrophs. The missing enzyme may also be needed elsewhere as the bacteria multiply in vivo. It has been established that besides the capsule, well-characterized macromolecules are also important for the pathogenesis of B. anthracis. These compounds had been designated as "aggressive" for about 50 years ago. They are an important factor in the course of the disease (Bail 1904). They are only indispensable in the in vivo multiplication; in vitro they are superfluous. Keppie, Harris-Smith and Smith (1963) thought that the capsule material (D-glutanic acid-polypeptide Ivanóvics and Bruchner 1937) also has an "aggression" effect. However, the individual components of anthrax-toxin are more important. Three components have been found for the toxin -all with protein characteristics- (Stanley and Smith 1961). Asperagine could be involved in the reaction sequences as as amino-group donor, as in the conversion of citrullin to arginine (Umbarger and Davis 1962).

The missing enzyme activity in the adenine-auxotrophs of B. anthracis can be replaced by adenine in vitro, but is not sufficient for the mutants to multiply in vivo. Further experiments are in progress in order to elucidate unknowns and strengthen these hypotheses.

Summary

A considerable difference exists between the virulences of purine auxotrophs of Bacillus anthracis due to the site of block in the purine biosynthetic pathway. All strains in which the block were situated in front on inosylic acid (hy⁻ auxotrophs) or in the conversion of xanthylic acid to guanylic acid (gu⁻ auxotrophs) were proved to be virulent.

Contrary, adenine auxotrophs in their phenotype appeared completely avirulent. Inoculated into mice, multiplication does not result even if

the animals are repeatedly treated with subtoxic amounts of adenine. The conditions for multiplication in vitro and in vivo are apparently different in these mutants. It may be assumed that the two enzymes involved in the conversion of inosyuic acid to adenylic acid (adenyl-succinate-synthase and adenyl-succinase), or one of them, also play a role in the synthesis of such cell components that are indispensable for in vivo multiplication.

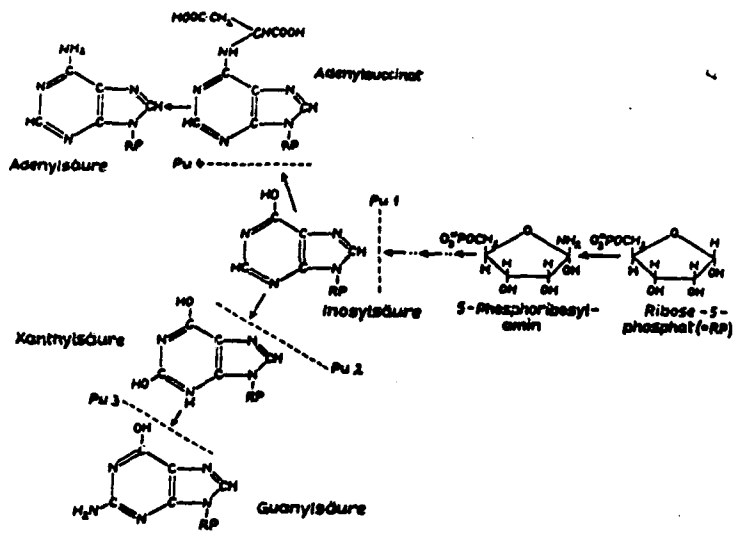

Abb. 1. Schema der Purin-Biosynthese und der den einzelnen Auxotrophen entsprechende Block.

Figure 1. Schema of purine-biosynthesis with the corresponding blocks for the auxotrophs.

Die ad⁻-Auxotrophen benötigten von der adaequaten Base etwas größere Mengen als die übrigen Purin-Auxotrophen. Einen Einblick in die Wachstums-Reaktion des einen Stammes bietet Abbildung 2.

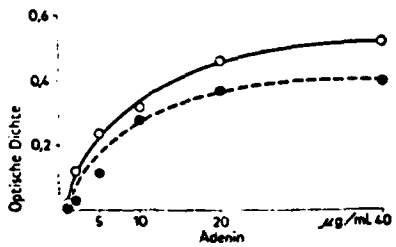

Abb. 2. Wachstum des 23 C-ad⁻-Stammes nach 24- und 48stündiger Inkubation bei verschiedenen Adenin-Konzentrationen und 37°C.
● = 24stündige Inkubation; ○ = 48stündige Inkubation.

Figure 2. Growth of strains 23C⁻ad⁻ after 24 and 48 hours of incubation (37°C) with different adennine concentrations.
● = 24 hours; ○ = 48 hours incubation

Tabelle 1

Wachstum des Stammes 61 C-hy⁻ nach 24 und 48 Std. in flüssigem Nährboden auf den Einfluß verschiedener Basen

Konz. µg/ml	Wachstumsgrad: optischer Densitätswert							
	Hypoxanthin		Xanthin		Adenin		Guanin	
	24 Std.	48 Std.	24 Std.	48 Std.	24 Std.	48 Std.	24 Std.	48 Std.
2,0	0,08	0,16	0,00	0,10	0,00	0,05	0,16	0,24
4,0	0,16	0,23	0,08	0,16	0,00	0,10	0,22	0,36
8,0	0,18	0,32	0,08	0,22	0,00	0,14	0,14	0,41
16,0	0,23	0,36	0,18	0,35	0,00	0,29	0,31	0,43

Im Falle der 24stündigen Inkubation bedeutete der 0,00-Wert in der Tat eine so geringgradige, mit freiem Auge wahrnehmbare Trübung, die nicht meßbar war.
In den keine einzige der Basen enthaltenden Kontrollröhrchen bestand auch nach 48 Std. noch keine sichtbare Trübung.

Table 1. Growth of strain 61C⁻hy⁻ after 24 and 48 hours in liquid medium; the influence of various bases.
(growth measured by optical density)
0.00 indicates growth was not measurable
No growth (turbidity) was obserfed in any control tubes after 48 hours.

Tabelle 2

Das Schicksal der mit den einzelnen Purin-Auxotrophen geimpften Mäuse und das Ergebnis der bakteriologischen Untersuchung der eingegangenen Tiere

Stamm	Zahl der infizierten Tiere	Zahl der verend. Tiere	Zahl der bakteriologisch untersuchten Tiere	Äußeres der Kolonien in der CO_2-Atmosphäre*	Nährbedarf**
6 C-ad-	26	0			
20 C-ad-	20	0			
23 C-ad-	5	0			
83 C-ad-	5	0			
2 C-gu-	10	8	7	3 M, 3 R, 1 G	G
3 C-gu-	5	4	3	1 M, 2 R	G
30 C-gu-	6	6	6	6 R	G
52 C-gu-	5	4	3	1 M, 2 R	G
66 C-gu-	3	1	1	M	G
68 C-gu-	3	2	2	1 M, 1 R	G
15 C-hy-	5	5	2	2 G	A, G, H, X
31 C-hy-	6	4	2	1 M, 1 R	A, G, H, X
39 C-hy-	5	4	2	2 G	A, G, H, X
58 C-hy-	10	10	8	2 M, 8 R	A, G, H, X
61 C-hy-	5	5	2	2 M	A, G, H, X
65 C-hy-	3	2	1	1 G	A, G, H, X
77 C-hy-	3	3	3	3 M	A, G, H, X

* Verteilung des Äußeren der Kolonien der isolierten Stämme:
M = mukoid
R = nicht mukoid
G = M + R-Kolonien gemischt

** Die Ansprüche der Isolate waren mit A = Adenin-, G = Guanin-, H = Hypoxanthin- und X = Xanthinbasen zu befriedigen.

Table 2. The fate of mice inoculated with purine-auxotrophs, and the result of the bacteriological investigations of the dead animals

Strain	# animals infected	# dead mice	# bact. sampled	extreme colonies in CO_2 atmos.	nutrion req.

* Distribution of the colonies of the isolated strains
M = mucoid; R = not mucoid; G = M R colonies mixed
** requirements of the isolated strains satisfied with;
A = adenine, G = guanine, H = hypoxanthine, X = xanthine

Tabelle 3

Die Virulenz der verschiedenen kapsulogenen Purin-Auxotrophen bei Mäusen nach subkutaner Injektion

Stamm	DLM	
	Nicht behandelt	Behandelte Tiere*
VC⁺ („wild")	2 × 10	nicht untersucht
23 C⁺ad⁺	2 × 10	nicht untersucht
23 C⁺ad⁻	> 2 × 10⁸	> 2 × 10⁸
6 C⁺ad⁻	> 2 × 10⁸	> 2 × 10⁸
20 C⁺ad⁻	> 10⁸	> 10⁸
83 C⁺ad⁻	> 10⁸	> 10⁸
61 C⁺hy⁻	2 × 10⁸	2 × 10⁸
5 C⁺hy⁻	2 × 10⁸	2 × 10⁸
58 C⁺hy⁻	2 × 10	2 × 10
30 C⁺gu⁻	2 × 10⁸; 2 × 10⁸	2 × 10
68 C⁺gu⁻	2 × 10⁸; 2 × 10⁸	2 × 10⁸

Die mit > bezeichneten Werte bedeuten, daß die mit der angegebenen Sporenzahl geimpften Tiere am Leben blieben und Infektionen mit größeren Dosen nicht vorgenommen wurden.

* Von den betreffenden Basen erhielten die Tiere zum Zeitpunkt der Infektion 1 mg und anschließend noch 4–6 mal je 0,5 mg intraperitoneal.

Figure 3. Investigations on the direction of the mutations of the Vollum strains, and the virulence of individual mutants. (The numbers represent the DLM (Min-lethal dose) of the individual mutants)

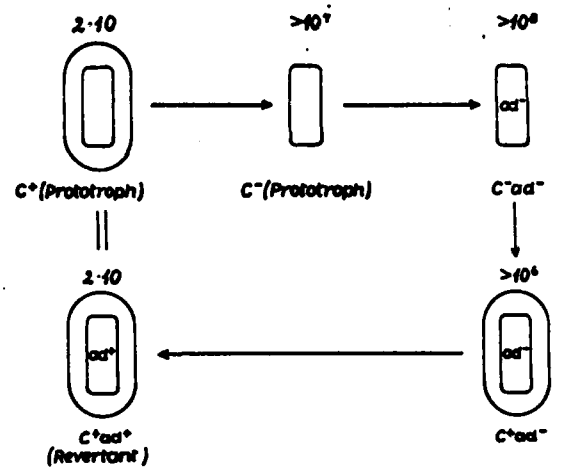

Abb. 3. Untersuchte Richtlinien der Mutation des Vollum-Stammes und die Virulenz der einzelnen Mutanten.

Die Ziffern stellen die DLM-Werte der einzelnen Mutanten dar.

Table 3. Virulence of various encapsulated purine-auxotrophs in mice, after subcutaneous injection.

Strains	Not Treated	Treated Animals

> means that the number of spores did not kill. To use a greater number was not planned.

* Animals received 1 mg of the particular bases at the time of infection; later 4-6 times 0.5 mg: all intraperitoneally.

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